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TI The search for novel adjuvants for early life vaccinations: can "danger" motifs show us the way?.

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SO Cellular Immunology (1998), 190(1), 77-82

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Article title	The influence of base sequence on the immunological properties of defined oligonucleotides
Article identifier	016231099800364X
Authors	Pisetsky_D_S Reich_C_F_III
Journal title	Immunopharmacology
ISSN	0162-3109
Publisher	Elsevier Netherlands
Year of publication	1998
Volume	40
Issue	3
Supplement	0
Page range	199-208
Number of pages	10
User name	Adonis
Cost centre	
PCC	\$22.00
Date and time	Thursday, March 13, 2003 9:52:29 PM

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The influence of base sequence on the immunological properties of defined oligonucleotides

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Accepted 27 July 1998

Abstract

To assess the influence of base sequence on the immunostimulatory activities of DNA, cell binding and mitogenicity of a series of 30-mer phosphodiester oligonucleotides were tested using murine spleen cells. These compounds consisted of either a single base or a six base CpG motif in the context of 5' and 3' flanking sequences of each base. Among fluoresceinated oligonucleotides, (dG)30 had the highest binding of single base compounds tested while the presence of dG flanks increased binding of compounds with six base motifs, whether active or inactive. In assays of mitogenesis including incorporation of thymidine and uridine as well as expression of cell surface CD69, (dG)30 induced the highest responses among single base compounds. Among compounds with an active six base motif, the extent of proliferation varied with flanking sequence, with dG flanks producing the greatest stimulation in all assays tested. Together, these findings indicate that a variety of base sequences may affect the immunomodulatory properties of DNA, with the activity of dG sequences perhaps resulting from the formation of variant DNA structures. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: DNA; Mitogenicity; Oligonucleotides; Immunostimulation; Lymphocytes

1. Introduction

DNA is a complex macromolecule whose immunological activities depend on sequence microheterogeneity (Pisetsky, 1996). Although mammalian DNA is immunologically inert, DNA from bacteria displays potent immunostimulatory properties that include polyclonal B cell activation as well as cy-

tokine induction. These properties result from DNA motifs that center on an unmethylated CpG dinucleotide which is flanked by two 5' purines and two 3' pyrimidines (Pu-Pu-CpG-Pyr-Pyr) (Tokunaga et al., 1984; Messina et al., 1991; Tokunaga et al., 1992; Yamamoto et al., 1992a,b; Krieg et al., 1995; Klinman et al., 1996; Sato et al., 1996). These immunostimulatory sequences (ISS) occur much more commonly in bacterial DNA than mammalian DNA because of differences in the patterns of base methylation; in addition, CpG occurs in mammalian DNA at frequencies lower than predicted, a phenomenon known as CpG suppression (Bird, 1987; Hergersberg, 1991). Because of its content of ISS, bacterial

Abbreviations: ISS, immunostimulatory sequences; AP, active palindrome

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DNA may serve as a trigger for innate immunity in the setting of infection (Pisetsky, 1996).

Although CpG motifs are immunologically active, other DNA sequences may induce immune cell activation or potentiate responses induced by ISS. In particular, oligonucleotides containing runs of deoxyguanosine (dG) display enhanced immunological activities (Messina et al., 1993; Pisetsky and Reich, 1993; Kimura et al., 1994). This effect may result from the variant DNA structures dG can form because of its ability to base pair with itself; these structures, known as quadruplex DNA, include four stranded arrays in either the parallel or antiparallel orientation (Tougard et al., 1973; Williamson et al., 1989; Sen and Gilbert, 1990). Thus, the inclusion of dG sequences in oligonucleotides bearing ISS increases induction of interferon- γ by murine spleen cells because of expression of IL-12. The potentiation of cytokine production results from increased oligonucleotide binding to macrophages that is mediated by the scavenger receptor (Kimura et al., 1994). This receptor has broad ligand specificity and can bind to a variety of macromolecules that include acetylated lipoproteins, dextran sulfate as well as certain polynucleotides such as poly dG (Brown et al., 1980; Krieger, 1992; Pearson et al., 1993; Krieger and Herz, 1994; Hughes et al., 1995).

The immunological effects of dG compounds extend to B cells, although these cells lack a scavenger receptor. As shown in *in vitro* cultures, poly dG as well as an 18-mer dG oligonucleotide are mitogenic for B cells under conditions in which other synthetic homopolymers as well as mammalian DNA are inactive (Pisetsky and Reich, 1993). Since ISS and dG oligonucleotides differ structurally, they may trigger distinct pathways of cell activation.

To explore further these mechanisms, we have assessed the cell binding and mitogenicity of a series of synthetic oligonucleotides comprised of either a single base or an ISS in the context of different single base flanking sequences. Results of these studies indicate that dG runs promote binding to lymphocyte cell surfaces, although the effects on mitogenicity vary depending on the ISS and flanking sequence. These findings define further the influence of base sequence on immunomodulatory properties of DNA and raise the possibility that DNA can trigger mitogenesis by more than one mechanism.

2. Materials and methods

2.1. Oligonucleotides

Oligonucleotides were synthesized by Midland Certified Reagent (Midland, TX), using cyanoethyl phosphoramidite chemistry and purified by gel filtration. Compounds purchased for fluorescein labeling or coupling to Sepharose beads were synthesized with a 5'-amino linker arm and 5-carbon spacer. Stock solutions were made by dissolving the dried oligonucleotides in distilled water and sterilizing by membrane filtration (Millex-GV, Millipore, Bedford, MA). O.D. 260 readings before and after filtration showed no detectable loss of oligo.

To fluoresceinate the oligonucleotides, compounds with amino linker arms were dissolved in carbonate/bicarbonate buffer (pH 9.5) at a concentration of 10 mg/ml. Fluorescein isothiocyanate (FITC), (Sigma) was added at a ratio of 0.1 mg FITC/10 mg oligonucleotide and reagents were allowed to react 2 h at room temperature protected from light. Unreacted FITC was removed from the labeled oligonucleotides by gel filtration through a Sephadex G-25 column (NAP10 Column, Pharmacia, Piscataway, NJ) followed by ethanol precipitation.

Immobilization of oligonucleotides on beads was performed using oligonucleotides synthesized with a 5' amino linker. These compounds were coupled to cyanogen bromide activated Sepharose 4B in PBS pH 8.0 at 4°C for 3 h. Unreacted sites were blocked with 1 M ethanolamine in PBS. Amount of unbound oligonucleotide was measured by O.D. 260 to determine efficiency of coupling.

2.2. Cellular assays

Single cell suspensions were prepared from spleens removed from BALB/c or C3H/HeJ mice (Jackson Laboratory, Bar Harbor). The spleens were pressed between microscope slides to free cells. Red blood cells were removed by hypotonic ammonium chloride lysis. Following three washings with serum free RPMI 1640 medium, (Sigma, St. Louis, MO), cell numbers were determined using a hemacytometer. For some experiments, splenocyte preparations

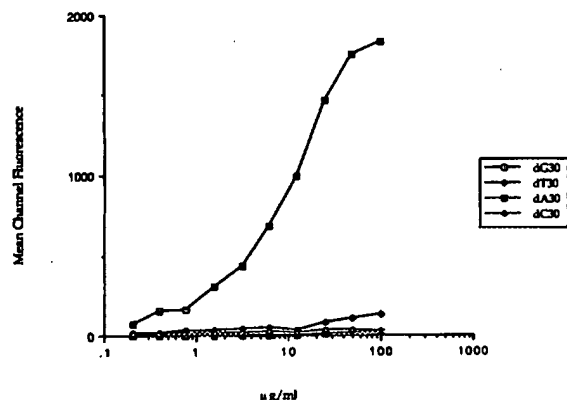


Fig. 1. Binding of oligonucleotides to spleen cells. 30-mer oligonucleotides were fluoresceinated and assessed for binding to spleen cell preparations as described in Section 2. Results are reported in terms of mean fluorescence at each concentration.

were enriched for B or T cells. B cell enrichment was accomplished by treatment of cells with anti-Thy1.2 (Sigma) followed by rabbit complement (Cedarlane Laboratories, Hornby, Ontario) to remove T cells. T cell enrichment was accomplished using a commercially available mouse T cell enrichment column following the protocol supplied by the manufacturer (Biotex Laboratories, Edmonton, Alberta). Efficiency of separation procedures was assessed by flow cytometry using phycoerythrin or fluorescein labeled antibodies to Thy1.2 (T cells) or B220 (B cells) obtained from Pharmingen (San Diego, CA).

For proliferation experiments, cells were washed twice and resuspended in RPMI 1640 with L-glutamine and sodium bicarbonate (Sigma) containing 5% heat inactivated fetal bovine serum (HyClone, Logan, UT), 200 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and 20 µg/ml gentamicin and plated in 96 well tissue culture plates (Costar, Cambridge, MA) at a concentration of 5×10^5 cells/well. Plates were incubated at 37° in 5% CO₂ in the presence of various additives. After 48 h, 0.5 µCi of H3-thymidine (NEN, Boston) or H3-uridine (NEN, Boston) was added to each well 6 h prior to harvest on glass fiber filters using a Bellco Microharvester (Bellco, Vineland, NJ). Incorporation was measured by liquid scintillation counting in a Packard Tri-Carb liquid scintillation counter (Packard, Downers Grove, IL). Results are expressed as the mean of triplicate wells.

2.3. Flow cytometry

Measurement of oligonucleotide binding was done with a FACSAN flow cytometer (Becton Dickinson, Mountain View, CA). The binding of FITC labeled oligos was performed with cells for 20 min on ice with or without competing ligand in HBSS. Cells were then washed $3 \times$ with HBSS and resuspended in HBSS. Cells were kept below 4° during this procedure. Propidium iodide (Sigma) at a concentration of 1 µg/ml was included in the first wash step to exclude the dead cell population. For some experiments, specific cell populations were identified using phycoerythrin labelled monoclonal antibodies to B220 or Thy1.2 (Pharmingen) or phycoerythrin labelled goat F(ab')₂ anti-mouse immunoglobulin (TAGO, Burlingame, CA).

To assess cell surface CD69 expression, cells were stimulated with oligo for 18–20 h in complete medium. Cells were harvested to tubes and incubated with FITC labeled anti-CD69 antibody (Clone H1.2F3, Pharmingen) for 10 min at room temperature and evaluated by FACSCAN analysis without fixation.

3. Results

3.1. Influence of oligonucleotide sequence on cell binding

To assess the influence of DNA sequence on lymphocyte binding and mitogenicity, a series of

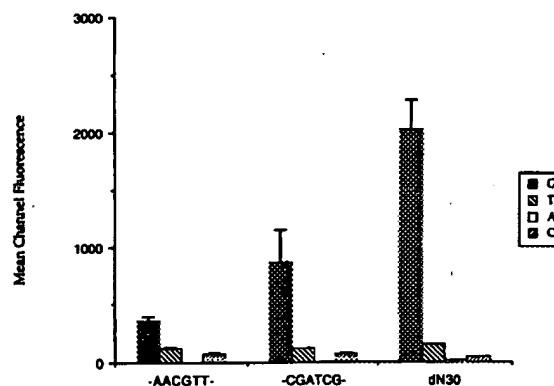


Fig. 2. Binding of oligonucleotides to spleen cells. 30-mer oligonucleotides containing six base motifs were tested for binding at a concentration of 10 µg/ml. Results are reported in terms of mean channel fluorescence.

Table 1
Binding of oligonucleotides to lymphocyte populations^a

Compound	Mean channel fluorescence	
	B cells	T cells
N12-AACGTT-N12		
N = A	7	4
N = C	159	7
N = G	445	265
N = T	137	40

^a Binding of fluoresceinated oligonucleotides to isolated B and T cell populations was assessed by FACS analysis. Results are presented in terms of mean channel fluorescence.

synthetic oligonucleotides varying in base sequence was tested by FACS analysis and in vitro proliferation assays. These compounds were all 30 nucleotides in length and consisted of either single bases or a six base core flanked on the 5' and 3' side by 12 nucleotide runs of each base. The six base core had a sequence predicted to be active (AACGTT) or inactive (CGATCG) in terms of models for the structure of mitogenic CpG motifs (i.e., Pu-Pu-CpG-Pyr-Pyr). The active sequence is designated AP since it can form a palindrome.

Since dG runs have increased interactions with macrophages (Kimura et al., 1994), we tested whether these sequences also had increased binding to lymphocyte cell surfaces. To assess this interaction, FACS analysis was performed using a series of

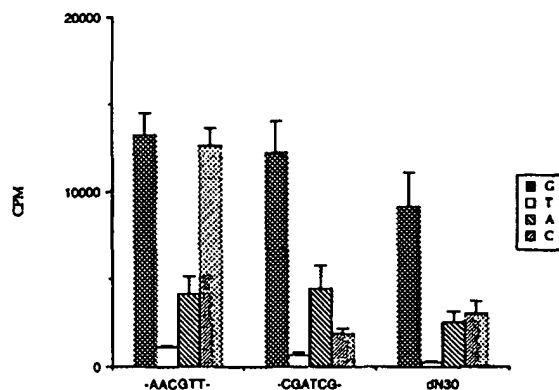


Fig. 3. Mitogenicity of oligonucleotides. Spleen cell cultures were stimulated with oligonucleotides at 50 μ g/ml and proliferation assessed after 48 h by thymidine incorporation. Each of the compounds tested was 30 bases long. dN30 compounds consisted of a single base while the other compounds had a six base core motif flanked on the 5' and 3' ends by 12 bases of each nucleotide. Results are reported in terms of mean (\pm standard deviation) of triplicate determinations.

directly fluoresceinated oligonucleotides. Fig. 1 shows the results of binding to BALB/c spleen cells of oligonucleotides containing single bases. As these results demonstrate, dG oligonucleotides display much higher levels of cell surface binding than any of the other three single base oligos. Furthermore, dG flanks produced higher levels of DNA surface binding than the other flanks in oligonucleotides

Table 2
Analysis of oligonucleotide binding by inhibition assays^a

Inhibitors		% Inhibition	
		FITC G12-AACGTT-G12	FITC G12-CGATCG-G12
N12-AACGTT-N12	= N		
	G	46 \pm 7	43 \pm 2
	T	0 \pm 0	4 \pm 3
	A	-9 \pm 6	-6 \pm 9
	C	18 \pm 6	1 \pm 10
N12-CGATCG-N12	G	59 \pm 3	54 \pm 3
	T	7 \pm 4	4 \pm 3
	A	7 \pm 5	-4 \pm 4
	C	19 \pm 5	27 \pm 4
N30	G	46 \pm 2	27 \pm 6
	T	0 \pm 4	-10 \pm 1
	A	-1 \pm 1	-1 \pm 9
	C	13 \pm 11	20 \pm 8

^a Inhibition binding assays were performed using as ligands two 30-mer fluoresceinated oligonucleotides with dG flanks. The concentration of ligands was 10 μ g/ml while inhibitors were tested at 200 μ g/ml. Percent inhibition was calculated on the basis of mean fluorescence.

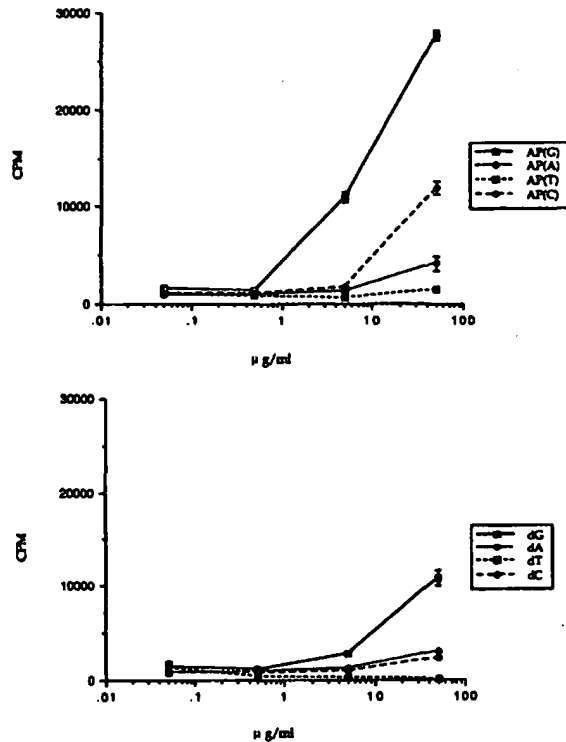


Fig. 4. Stimulation of thymidine uptake by oligonucleotides. Spleen cell preparations were stimulated with oligonucleotides at concentrations indicated. The oligonucleotide tested had an active six base motif designated as AP since it can form a palindrome. After 48 h, thymidine incorporation was assessed. Results presented are the means of triplicate determinations.

containing either active or inactive motifs (Fig. 2). This pattern was observed with both B as well as T cell populations (Table 1).

To evaluate further the interaction of DNA oligonucleotides with cells, competitive binding assays were performed using as ligands 30-mer oligonucleotides containing an active or inactive motif in the context of (dG)₁₂ flanks. As the data in Table 2 show, the binding of these compounds to spleen cells was inhibited by oligonucleotides containing extended runs of dG, either in the presence or absence of the 6 base motifs. Oligos containing dC runs also produced inhibition of binding, although these compounds had much lower levels of surface binding than dG compounds when assessed by direct binding assays. In contrast, dA and dT compounds were inactive as inhibitors. In conjunction with results of the direct binding assays, these results show that the

interaction of oligonucleotides with cells varies with sequence, with the presence of dG structures leading to markedly increased levels of surface binding.

3.2. Influence of DNA sequence on mitogenicity

The mitogenicity of these compounds was next evaluated to assess the relationship between cell binding and capacity to induce proliferation. As noted by other investigators, thymidine incorporation may not accurately reflect the activities of dT containing oligonucleotides since nuclease digestion may release thymidine and cause dilution of intracellular pools (Matson and Krieg, 1992; Mouthon et al., 1994). Thymidine assays may nevertheless be used for comparison of the activities of other compounds. To provide a more complete assessment of mitogenesis, we also measured incorporation of uridine as well as cell surface expression of CD69. Recent studies have demonstrated that this marker provides

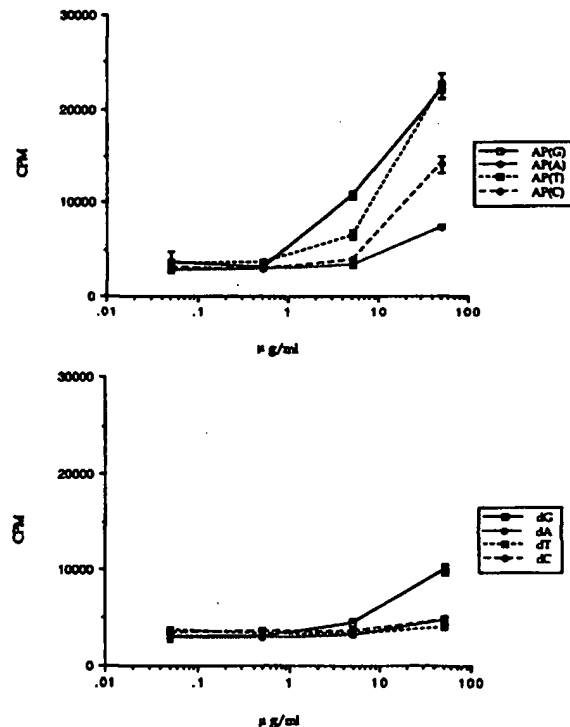


Fig. 5. Stimulation of uridine uptake by oligonucleotides. Spleen cell preparations were stimulated with oligonucleotides at concentrations indicated. After 48 h, uridine uptake was assessed. Results presented are the means of triplicate determinations.

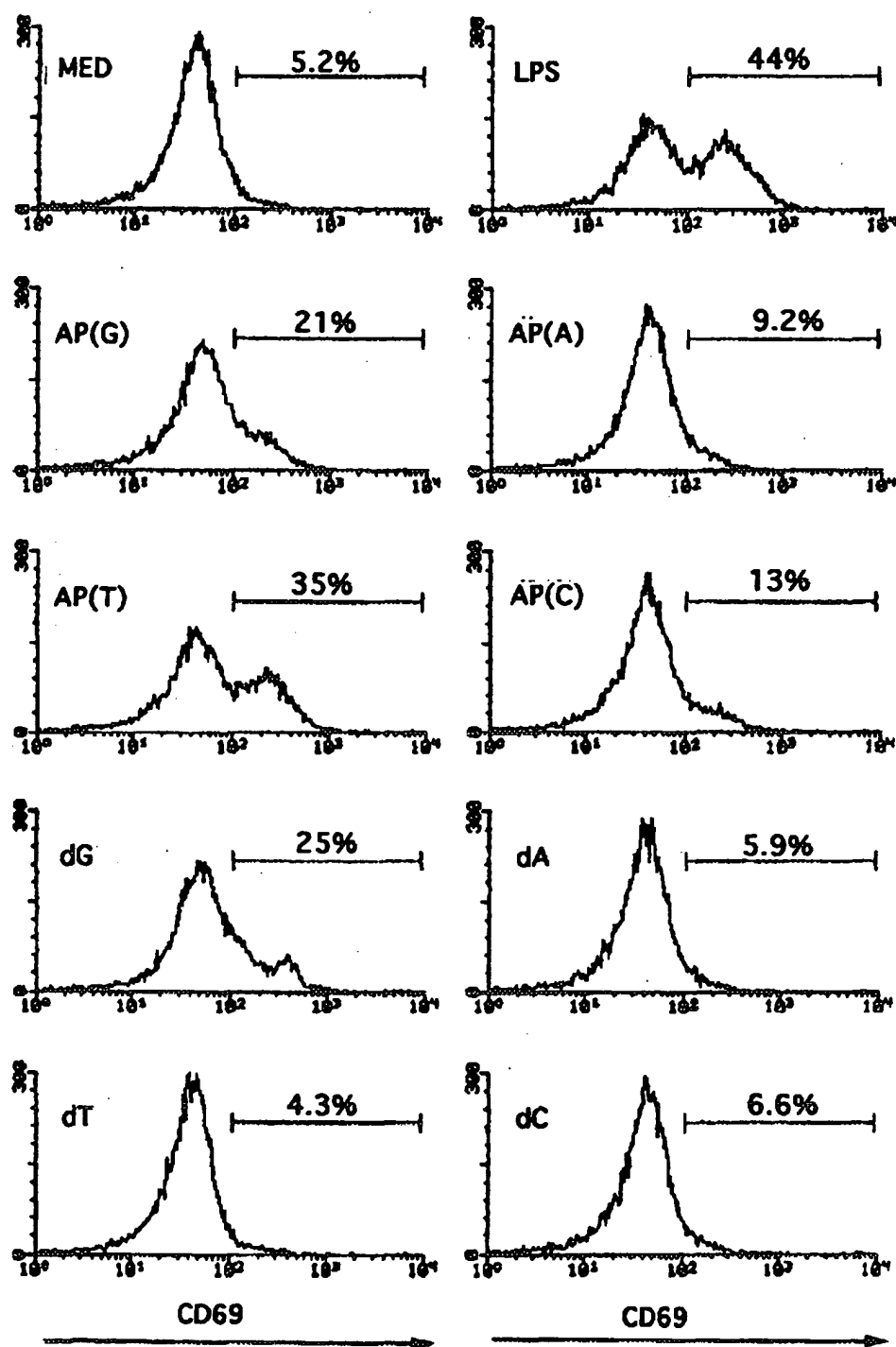


Fig. 6. Stimulation of cell surface CD69 expression by oligonucleotides. Spleen cells were stimulated in culture with oligonucleotides at a concentration of 50 $\mu\text{g}/\text{ml}$. CD69 was measured by flow cytometry as described in Section 2.

an accurate measure of B cell activation (Sun et al., 1997).

As shown in Fig. 3, among single base oligonucleotides, (dG)30 induced greater thymidine incorporation than other compounds, confirming observations that a CpG motif is not required for the induction of proliferation by synthetic oligonucleotides. Furthermore, the mitogenicity of the active motif (AACGTT) varied depending on the context of the flanking bases. Whereas this motif in the context of dG and dC bases produced significant thymidine incorporation, it was much less active in the context of dT and dA bases and induced incorporation similar to an inactive CpG motif. Furthermore, an oligonucleotide containing an inactive motif in the context of dG flanks was mitogenic. As shown in dose response experiments, dG flanks also caused stimulation at lower oligonucleotide concentrations (Fig. 4). Experiments with isolated B and T cell populations indicated that, for all oligonucleotides, only B cells show a mitogenic response (data not shown).

The assessment of mitogenicity by incorporation of uridine and the measurement of cell surface CD69 expression produced a somewhat different picture. Thus, among synthetic oligonucleotides containing a single base, only (dG)30 induced significant incorporation of uridine (Fig. 5). These results are similar to those with thymidine as well as previous studies. In contrast, among synthetic oligonucleotides contain-

ing an active six base sequence, dT, dC as well as dG flanks all led to significant incorporation of uridine whereas an oligonucleotide with dA flanks was inactive. Together, these findings indicate that the mitogenicity of an active motif varies with the context of flanking bases, with dA compounds inactive when tested by any assay. Measurement of cell surface CD69 expression produced results similar to that of uridine incorporation (Fig. 6). While substantiating the impact of flanking sequences on the activity of CpG motifs, these findings show that assessment of mitogenicity depends on the assay technique used.

3.3. Activity of oligonucleotides immobilized on beads

Oligonucleotides containing exclusively dG differ structurally from ISS containing CpG motifs. Furthermore, these compounds have dramatically enhanced cell surface binding. We therefore investigated the possibility that dG could induce proliferation by direct interaction with a cell surface receptor rather than following internalization. To test this possibility, the activity of dG oligonucleotides bound to Sepharose beads was assessed. As data in Fig. 7 indicate, dG on beads failed to induce mitogenesis under conditions in which soluble dG oligonucleotides were active. These findings suggest that dG oligonucleotides, similar to ISS, require internalization for activity despite their enhanced capacity to bind to cell surface.

4. Discussion

The data presented herein indicate that the immunomodulatory properties of DNA result from at least two types of sequences: ISS that center on unmethylated CpG motifs and extended runs of dG residues. The effects of these sequences, however, may vary depending on cell type. Whereas CpG motifs stimulate both B cell mitogenesis and IFN- γ production, dG runs stimulate only mitogenesis (Pisetsky and Reich, 1993; Kimura et al., 1994; Krieg et al., 1995). Furthermore, dG sequences lead to enhanced cell surface binding on B and T cells as shown herein as well as macrophages (Kimura et al., 1994). Together, these findings point to a unique

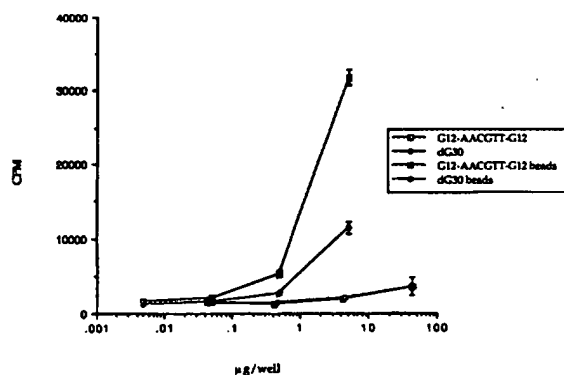


Fig. 7. Mitogenicity immobilized oligonucleotides. The mitogenicity of dG30 as well as G12-AACGTT-G12 was tested using either soluble oligonucleotides or oligonucleotides immobilized on Sepharose beads. Concentrations are indicated for oligonucleotides whether free or attached to beads. Results are reported in terms of mean (\pm standard deviation) of thymidine incorporation.

potential of dG residues for interaction and stimulation of murine lymphocytes.

The activity of dG compounds most likely reflects their structure and ability to assume helical arrays different from duplex B DNA. Because dG residues form non-conventional hydrogen bonds with each other, extended runs of dG can lead to intrastrand and interstrand interactions and the formation of four stranded arrays called quadruplex DNA. These four strand arrays can be assembled with DNA chains in the parallel or anti-parallel orientation (Williamson et al., 1989; Sen and Gilbert, 1990). While the functional role of quadruplex DNA is unknown, this variant structure may serve as an element in either gene regulation or chromosomal assembly.

As shown with both lymphoid and non-lymphoid cells, DNA appears to enter cells by different mechanisms depending on concentration. At low concentrations, DNA entry may reflect receptor mediated endocytosis whereas at high concentrations fluid phase endocytosis or potocytosis may be predominant. Using both polymeric DNA as well as synthetic oligonucleotides as ligands, surface receptors for DNA have been identified on many cell types. These receptors are proteins that vary in molecular weight. In general, these receptors are thought to bind DNA irrespective of base sequence (Bennett et al., 1985; Yakubov et al., 1989; Krieg et al., 1991; Akhtar and Juliano, 1992; Loke et al., 1992; Vlassov et al., 1994).

In contrast to these receptors, the macrophage scavenger receptor has marked preference for certain nucleic acids. This receptor binds a variety of macromolecules, including acetylated lipoproteins, dextran sulfate and fucoidan, at high affinity. While unable to bind natural DNA, the receptor can interact with defined polynucleotides that differ in structure from conventional helical DNA (e.g., quadruplex DNA) (Brown et al., 1980; Krieger, 1992; Pearson et al., 1993; Krieger and Herz, 1994). Because of the specificity of these interactions, dG oligonucleotides bind much better to macrophages than other oligonucleotides and most likely achieve higher intracellular concentrations.

These considerations suggest that, for cytokine production, the magnitude of the response reflects the quantity of CpG ISS DNA sequences inside IL-12-producing macrophages. These responses can

be enhanced by sequences (i.e., dG runs) that promote binding to the scavenger receptor. Similarly, agents that non-specifically promote DNA entry (i.e., cytofectins) can increase the effects of ISS. These lipid agents can adhere to DNA and facilitate uptake and access to the nuclear compartment (Felgner and Ringold, 1991; Behr, 1994; Yamamoto et al., 1994).

For B cells, dG sequences may play more than one role. Among single base oligonucleotides, only (dG)₃₀ induced significant proliferation when assessed either using thymidine or uridine incorporation as well as expression of cell surface CD69. Furthermore, this compound shows the highest level of cell surface binding when tested by FACS; the presence of dG flanks also increases the binding of six base sequences, either active or inactive. The surface binding of the dG oligos may result from a protein or DNA receptor with dG preference or a protein-independent interaction with membrane lipid. Thus, as shown using 10-mer phosphorothioate oligonucleotides, dG compounds bind better to liposome preparations than other single base oligonucleotides (Hughes et al., 1994).

Despite the enhanced binding to cell surfaces, dG compounds, like ISS, nevertheless appear to require internalization for activity. Thus, dG on Sepharose beads failed to stimulate proliferation whereas soluble dG was mitogenic. These observations resemble findings with CpG oligonucleotides and contrast with studies on the stimulation of human B cells by phosphorothioate oligonucleotides. With human cells, phosphorothioate oligonucleotides on Sepharose are mitogenic (Liang et al., 1996). The difference between human and murine responses could reflect species differences in the response to oligonucleotides; although murine cells respond to both phosphorothioate and phosphodiester compounds, human cells respond only phosphorothioates. Furthermore, the sequences active for human lymphocytes differ from the CpG motifs established for murine cells (Liang et al., 1996).

Assessing the impact of DNA sequences on mitogenicity can be problematic, however, because nuclease digestion can release nucleotides that dilute label pools or otherwise affect intracellular metabolism. Thus, dT oligonucleotides fail to induce thymidine incorporation, an effect resulting most likely from dilution of H3 thymidine by cold thymi-

dine from digested oligonucleotides (Matson and Krieg, 1992; Moulton et al., 1994). dT oligonucleotides containing an ISS can nevertheless stimulate uridine incorporation as well increase surface expression of CD69. The effects of digested oligonucleotides are likely complex as shown by the differences in the relative potency of various compounds measured using uridine and thymidine incorporation. In this regard, oligonucleotides containing dA flanks were inactive with any assay, perhaps resulting from metabolic effects of high concentrations of adenosine (Tattersall et al., 1975). Although the effects of digested oligonucleotides have been most apparent with synthetic compounds, they theoretically could occur with naturally occurring DNA as well. Evaluation of immunomodulatory effects of DNA may therefore entail the use of a variety of activation assays, including assays not dependent on label incorporation.

Among oligonucleotides containing single bases, (dG)₃₀ showed both the highest levels of cell binding as well as the greatest stimulation of proliferation tested with either thymidine or uridine. In contrast, among compounds with active 6 base motifs, cell binding was not directly related to the extent of proliferation observed when using uridine incorporation of CD69 expression. These findings suggest that DNA uptake may vary with time and concentration allowing CpG compounds with different flanks to show similar mitogenic activity despite differences in initial cell binding. In this regard, the response of B cells to CpG motifs appears to differ from that of macrophages and monocytes. In these cells, cytokine production was closely correlated with cell binding via the macrophage scavenger receptor. We have preliminary results that B cells and macrophages also differ in the extent to which lipofectin reagents enhance responses to bacterial DNA, suggesting that cell entry and signaling pathways differ in these cell types (Reich and Pisetsky, preliminary results).

Given the structural differences between CpG DNA and dG DNA, these sequences may trigger cells by different mechanisms. Quadruplex DNA, because of its surface interaction, may achieve high intracellular concentrations. This compound may be stimulatory because of the effects of high charge arrays in the nuclear compartment. This effect has been demonstrated with direct injection of other

polyanions into the nucleus of cells (Arnold et al., 1972; Smith et al., 1990).

In considering the effects of dG on B cells, it is important to note that, unlike CpG motifs, dG runs occur commonly in the mammalian chromosome and are a feature of telomeric DNA. These sequences may be immunologically inactive in natural DNA because of transience or low concentration. Alternatively, the presence of nucleosomal proteins may affect the display of this conformation. While the immune potential of dG sequences in natural DNA requires further evaluation, these findings nevertheless demonstrate the important impact of DNA sequence on lymphocyte binding and activation. Studies are therefore in progress to define the role of sequence on intracellular trafficking of DNA and localization in cell compartments.

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